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Histochemical analysis of collagen reorganization at the invasive front of oral squamous cell carcinoma tumors

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Abstract

Aim: The aim of the present study was to: (a) analyze the nature of collagen with respect to cohesive and discohesive invasive tumor front (ITF) of oral squamous cell carcinoma (OSCC); (b) analyze the clinicopathological correlation with the nature of collagen at the ITF of OSCC; and (c) correlate the nature of collagen with Broder's and Bryne's histological grading system of OSCC.

Methods: Tissue sections of 29 OSCC with ITF were stained with hematoxylin-eosin and picrosirius red staining for evaluation under a polarized microscope.

Results: Tumors with a cohesive front had a thick collagen fiber, are predominantly organized, red-yellow in color, well packed, and show strong birefringence ($P<.005$). A gradual change in the nature of the collagen fiber was observed in the discohesive tumor front, where the collagen fibers were thin, disorganized, yellow-orange to green-yellow in color, loosely packed, and with weak birefringence ($P<.005$).

Conclusion: A cohesive tumor front with organized collagen fibers prevents tumor invasion and metastasis. As it is inhibits an increase in tumor size, it is associated with the initial stage of tumor (I & II), whereas in a discohesive tumor front, the fibers might enhance the movement of tumor cells, resulting in invasion and metastasis.

KEYWORDS

cohesive tumor front, discohesive tumor front, invasive tumor front, oral squamous cell carcinoma, picrosirius red

1 | INTRODUCTION

Oral squamous cell carcinoma (OSCC) represents the majority of malignant lesions of the oral cavity, particularly in developing countries, where large populations are exposed to carcinogens, such as tobacco, smoke, and betel nut extracts.

OSCC is composed of two distinct components: (a) the malignant epithelial cells; and (b) the stroma in which they are dispersed.[1] One of the major aspects of tumor cell invasion and metastasis is the interaction between cancer cells and the extracellular matrix (ECM) component.[2] The ECM is composed of a ground substance made up of proteoglycans, glycoproteins, and water, and the fibrous component, including collagen and elastic fibers.[1] The ECM component is the key factor for nutrition and growth to any tumor, and also acts as barrier for the spread of the tumor.[3] Alterations in the ECM can play a role in recurrence and in the facilitation of tumor cell invasion.

Collagen constitutes 34% of the total ECM proteins and forms the integral part of connective tissue stroma. It plays a vital role in maintaining structural integrity and in determining tissue function. The collagenous tissue in the stroma gives strength to the tumor by giving it a skeleton. An increase in collagen content of the ECM increases the mechanical stiffness and transport resistance of tumors.[4] In a recent study by Li et al.,[5] it was found that collagen fiber plays an important role in ECM destruction and remodeling, so the study of interstitial collagen has been the mainstay of investigative histological procedures in understanding the pathogenesis of the lesion.

Collagen has natural birefringence, which is attributed to the arrangement of its fibers, and is enhanced by staining, such like Van

Gieson's, Masson's trichrome, and picrosirius red (PSR). PSR staining is considered a highly-specific and selective stain for collagen fibers due to its ability in differentiating between different types of collagen fibers in various pathological conditions.

There are limited histochemical studies in the literature on methods to detect, quantify, and analyze the collagen in the invasive tumor front (ITF) of OSCC.

1.1 | Aims

The aim of the present study was to: (a) analyze the nature of collagen with respect to cohesive and discohesive invasive tumor front (ITF) of oral squamous cell carcinoma (OSCC); (b) analyze the clinicopathological correlation with the nature of collagen at the ITF of OSCC; and (c) correlate the nature of collagen with Broder and Bryne's histological grading system of OSCC.

2 | MATERIALS AND METHODS

The scientific and ethics committee of Sri Dharmasthala Manjunatheshwara College of Dental Sciences and Hospital (Dharwad, Karnataka, India) approved the present study. Twenty-nine histologically-diagnosed cases of OSCC and two controls of normal mucosa were retrieved from the archives of Department of Oral Pathology (SDMCDSH, India). All of the sections were subjected to hematoxylin-eosin and PSR staining, and Sirius Red F3B (Avinash Chemicals, Bengaluru, Karnataka, India). Following deparaffinization and hydration in distilled water, the sections were incubated in .1% (w/v) Sirius red F3B in saturated picric acid solution for 1 hour at room temperature.

All of the patients underwent radical surgery with the removal of lymph nodes conducted at SDMCDSH from 2009 to 2015.

The majority of the OSCC cases showed a discohesive tumor front (n=19, 63%), which was represented by high degree of tumor cell dissociation, followed by cohesive tumor front (n=11, 37%).

2.1 | Inclusion and exclusion criteria

The inclusion criterion was OSCC patients who underwent radical surgery with the removal of lymph nodes. The exclusion criteria were incisional biopsies only, patients with a lack of clinical details, and areas of necrosis.

2.2 | Collagen analysis

All of the OSCC PSR stained sections were analyzed under a polarizing microscope by three blinded observers to note the nature of the collagen (i.e. thickness, organization, hue, density, and birefringence), irrespective of whether the tumor was cohesive or discohesive at the ITF. Collagen fiber nature was analyzed under the corresponding grades of carcinoma for Broder and Bryne's grading system. Data of each case was tabulated and were subjected for statistical analysis.

2.3 | Statistics

Pearson's correlation test was employed for comparisons between various parameters in the different groups.

3 | RESULTS

The categorical distribution of clinicopathological features of the 29 OSCC patients showed a male predominance (83%), with equal age distribution above and less than 45 years (50%) of tobacco chewing (63%). The tumor features included: (a) size mainly <4 cm (59%) on the buccal mucosa, retromolar trigone, and buccogingival sulcus (76%); (b) tumor, node and metastasis (TNM) classification stages I and

TABLE 1 Categorical distribution of clinicopathological features of oral Squamous cell carcinoma patients

Parameter	Category	No. cases	%
Sex	Male	24	83
	Female	5	17
Age (years)	<45	14	50
	>45	15	50
Habit	Tobacco	18	63
	Smoking	5	17
	Combination	6	21
Tumor size	<4 cm	17	59
	>4 cm	12	41
Site	BM, RMT, BGS	22	76
	T	6	20
	GIN, ALV	1	4
TNM stage	I+II	17	57
	III+IV	12	43
Broder grading	Well-differentiated carcinoma	20	67
	Moderately-, moderately-to-poorly differentiated carcinoma	9	33
IFG grading	4-8	2	10
	9-12	11	37
	13-16	16	53
Pericapsular invasion	Negative (n=0)	21	73
	Positive (n=1)	8	27
Surgical margins	Negative (n=0)	25	87
	Positive (n=1)	4	13
Pattern of invasion	Cohesive	10	37
	Discohesive	19	63

ALV, alveolus; BGS, buccogingival sulcus; BM, buccal mucosa; GIN, gingiva; IFG, invasive front grading; n=number of cases; RMT, retromolar trigone; T, tongue; TNM, tumor, node, and metastasis.

II (57%); (c) well differentiated, according to Broder's grading (67%), (d) an Invasive Front Grading (IFC) score of 13-16 (53%), with the absence of pericapsular invasion (73%); (e) negative surgical margins (87%); (f) and a discohesive tumor front (63%) (Table 1). In the present study, we found a positive correlation (P<.01) between collagen fiber nature in relation to patterns of invasive front. Tumors with a cohesive front showed thick collagen fibers, organized, red-yellow in color, well packed, and with strong birefringence (Figure 1). A gradual change in collagen fiber nature was observed in the discohesive tumor front, where the collagen fibers were thin, disorganized, yellow-orange to green-yellow in color, loosely packed, and with weak birefringence (Figures 2, 3, and 4) (Table 2).

3.1 | Collagen fiber density

When collagen fiber density was compared with the size of the tumor, the result was statistically significant (P<.05). In <50% of lesions <4 cm in size, the collagen fibers were well packed. In comparison, in more >80% of lesions >4 cm in size, the collagen fibers were loosely packed (Table 3).

Collagen fiber density was also statistically significant (P<.05) for TNM stage and lymph node metastasis. In >50% of OSCC, the dense, well-packed collagen fibers in the PSR-stained sections were TNM stages I and II, with the absence of lymph node metastasis. In comparison, in >80% of cases, collagen fibers were loosely packed, had TNM stages III and IV, and evident metastasis. Of all the parameters, density of collagen fibers showed maximum correlation with clinicopathological features (Table 3).

3.2 | Clinicopathological consideration

None of the other clinicopathological parameters showed a correlation with the advancing tumor front and nature of collagen fibers (P>.05), which could be due to small sample size, with the exception of tumor size, TNM staging, and lymph node metastasis. Collagen fiber density showed well-packed collagen fibers in tumors <4 cm (53%) in size, and loosely-packed collagen fibers in tumors >4 cm (83%). Similarly, well-packed fibers predominated in TNM stages I and II (59%), with absent metastasis (55%), and loosely-packed fibers predominated in

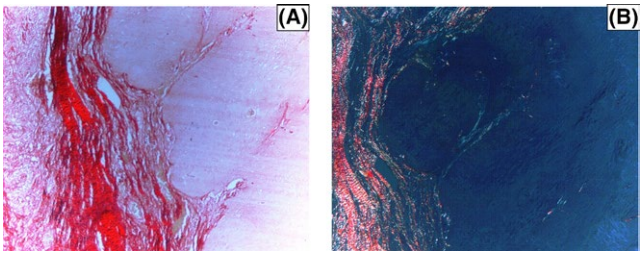

FIGURE 1 Photomicrographs of cohesive tumor front showing thick parallel bands of collagen fibers, which are (A) well organized (picrosirius red staining without polars, 10×) and (B) well organized with red-orange color birefringence (picrosirius red staining with polars, 10×)

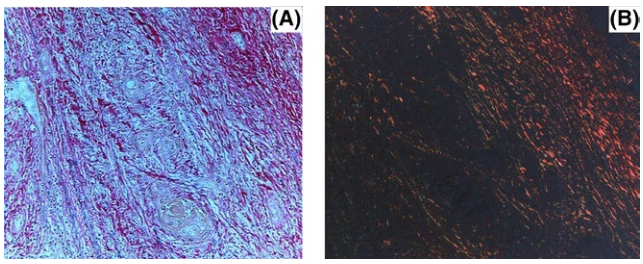

FIGURE 2 Photomicrographs of discohesive tumor front showing thin collagen fibers (A) around tumor islands (picrosirius red staining without polars, 10×) and (B) with red-orange color birefringence (picrosirius red staining with polars, 10×)

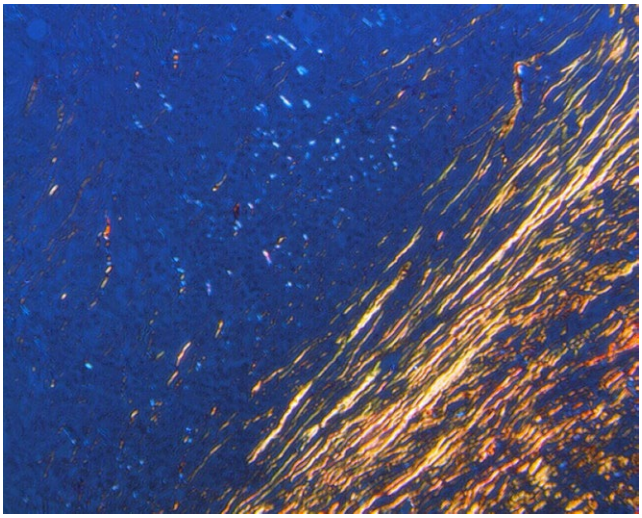

FIGURE 3 Photomicrograph of discohesive tumor front showing thin collagen fibers with yellow-orange color birefringence (picrosirius red staining with polars, 10×)

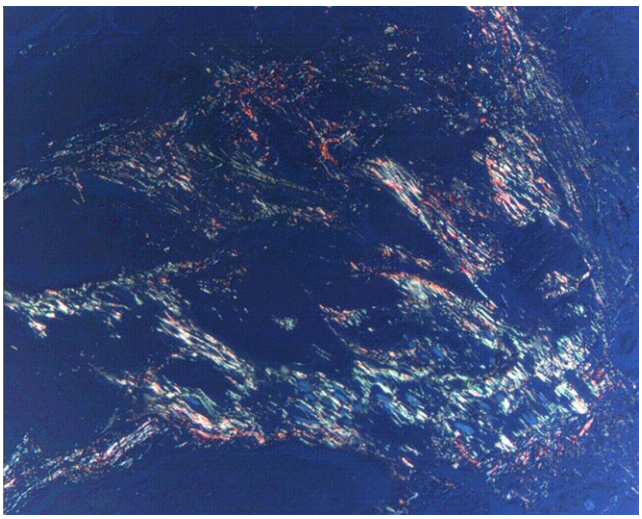

FIGURE 4 Photomicrograph of discohesive tumor front showing loosely-packed, thin, disorganized collagen fibers with greenish-yellow color birefringence (picrosirius red staining with polars, 4×)

TABLE 2 Nature of collagen fiber in relation to patterns of Invasive Tumor Front (ITF)

Parameter	Categories	Cohesive n=10 (%)	Discohesive n=19 (%)	*P*-value*
Thickness	Thick	11 (100)	4 (21)	.000
	Thin	0 (0)	15 (79)	
Organization	Organized	10 (91)	0 (0)	.000
	Disorganized	1 (9)	19 (100)	
Colors	Red-orange	11 (100)	3 (16)	.000
	Yellow-orange	0 (0)	3 (16)	
	Green-yellow	0 (0)	13 (68)	
Density	Well packed	10 (91)	2 (12)	.000
	Loosely packed	1 (9)	17 (88)	
Birefringence	Strong	11 (100)	5 (26)	.000
	Weak	0 (0)	14 (74)	

*Highly significant (<.01).

TABLE 3 Clinicopathological correlation with collagen fiber nature

Collagen fiber nature	Tumor size			TNM stage			Metastasis		
	<4 cm	>4 cm	*P*-value	I, II	III, IV	*P*-value	Absent	Present	*P*-value
Thickness									
Thick	8 (47%)	6 (50%)	.876	9 (53%)	6 (46%)	.713	11 (55%)	4 (44%)	.599
Thin	9 (53%)	6 (50%)		8 (47%)	6 (54%)		9 (45%)	5 (56%)	
Organization									
Organized	6 (35%)	3 (25%)	.555	7 (41%)	3 (23%)	.297	8 (40%)	2 (22%)	.351
Disorganized	11 (65%)	9 (75%)		10 (59%)	9 (77%)		12 (60%)	7 (78%)	
Color									
Red-orange	8 (47%)	5 (42%)	.883	8 (47%)	6 (46%)	.921	9 (45%)	5 (56%)	.837
Orange-yellow	2 (12%)	1 (8%)		2 (12%)	1 (8%)		2 (10%)	1 (11%)	
Yellow-green	7 (41%)	6 (50%)		7 (41%)	5 (46%)		9 (45%)	3 (44%)	
Density									
Well packed	9 (53%)	2 (17%)	.047*	10 (59%)	1 (15%)	.016*	11 (55%)	1 (11%)	.026*
Loosely packed	8 (47%)	10 (83%)		7 (41%)	11 (85%)		9 (45%)	8 (89%)	
Birefringence									
Strong	10 (59%)	5 (42%)	.362	12 (71%)	3 (31%)	.030*	11 (55%)	4 (44%)	.599
Weak	7 (41%)	7 (58%)		5 (29%)	9 (69%)		9 (45%)	5 (55%)	

**P*-value is significant (<.05).

TNM stages III and IV (85%) in cases of local lymph node metastasis (89%) (Tables 3 and 4).

3.3 | Histological grading system

The results were not statistically significant with Broder and Bryne's grading system of OSCC, but we found a significant correlation. The majority of cases (>50%), which were graded as poor-to-moderately differentiated, according to Broder and Bryne's grading system, showed collagen fibers at the advancing tumor front as disorganized, loosely packed, greenish-yellow in color, and weak birefringence(Table 4).

4 | DISCUSSION

One of the most prevalent keratinocyte-based cancers is squamous cell carcinoma. The highly-invasive and metastatic nature of OSCC makes it one of the most frustrating cancers to treat.[6] For a tumor cell to be invasive, it must be able to penetrate and move through the stroma.[7] The process of tumor dissemination involves specific interactions with tumor cell-surface adhesion receptors and multiple adhesive components of the ECM.

Tumor stroma plays a critical role in carcinogenesis. To grow beyond a minimal size of 1-2 mm, the tumor requires stroma.[4] The ECM

TABLE 4 Correlation between histological grading and collagen fiber nature at Invasive Tumor Front (ITF)

	Broder grading			Bryne's IFG Score			
Collagen fiber nature	**Well-differentiated carcinoma**	**Moderately-to-poorly differentiated carcinoma**	***P*-value**				***P*-value**
Thickness				**4-8**	**9-12**	**13-16**	
Thick	10 (47%)	5 (55%)	.782	1 (50%)	5 (45%)	8 (50%)	.972
Thin	10 (53%)	4 (45%)					
Organization							
Organized	8 (36%)	2 (22%)	.351	1 (50%)	6 (55%)	8 (50%)	.686
Disorganized	12 (64%)	7 (88%)		1 (50%)	4 (36%)	4 (25%)	
Colors							
Red-orange	11 (52%)	3 (33%)	.537	1 (50%)	7 (64%)	12 (75%)	.224
Yellow-orange	2 (11%)	1 (11%)		1 (50%)	6 (54%)	6 (37%)	
Green-yellow	7 (37%)	5 (56%)		1 (50%)	0	2 (13%)	
Density				**4-8**	**9-12**	**13-16**	
Well packed	9 (42%)	3 (33%)	.657	1 (50%)	5 (45%)	8 (50%)	.934
Loosely packed	11 (58%)	6 (67%)					
Birefringence							
Strong	11 (58%)	3 (33%)	.225	1 (50%)	6 (55%)	8 (50%)	.198
Weak	8 (42%)	6 (67%)		1 (50%)	4 (36%)	4 (25%)	

is composed of a ground substance made up of proteoglycans, glycoproteins, and water, and the fibrous component, including collagen and elastic fibers.[1] It provides vascular supply, nourishment, and oxygen supply, and limits the influx of inflammatory cells, thereby acting as a barrier for immunological rejection. The fibrous component of stroma has been shown to be associated with OSCC, and thus, stromal changes indicating the propensity of tumor cells to infiltrate and metastasize are now being studied as a prognostic indicator.[1]

Van Gieson's and Masson's trichrome staining might not be ideal for collagen fiber detection, as both of these methods fail to reveal thin collagen fibers, and the stains have a tendency to fade. These disadvantages lead to underestimation of the collagen content. This perplexing issue incited Puchtler et al. to seek a better method, and they found that Sirius Red dissolved in a saturated picric acid solution (PSR) consistently stained thin collagen fibers, did not fade, and was appropriate for use with polarized light microscopy.[8]

A combination of Sirius Red and picric acid was first considered to be a special stain for connective tissue in 1964, especially for differentiating the collagen subtype. It works on the principle that a sulfonic group of Sirius Red reacts with basic groups in collagen molecules; 126 Sirius dye molecules bind to purified collagen types I, II, and III. The enhanced birefringence of collagen is due to the attachment of the elongated dye molecules parallel to the long axis of the collagen. The orientation of the fibers to polars and collagen birefringence provide brightness to the collagen. Thickness, density of packing, and spatial arrangement determine the polarization birefringence of the collagen. PSR staining thus helps in better understanding collagen function and pathology.[9-12] In addition, when observed under a polarizing microscope, the PSR sections showed birefringence due to the anisotropic properties of collagen.[13,14]

As proposed in different studies, different patterns of invasion (representing different grades of tumor cell dissociation) are associated with prognostic cancer outcome in.[15] In the present study, we assessed the nature of collagen at the ITF of OSCC using PSR and a polarizing microscope.

In the present study, the cohesive tumor front with densely-packed distinct collagen showed reddish-orange colors, which were mainly concentrated around tumor islands, probably to prevent the tumor invasion. This could be due to the deposition of type 1 collagen fibers, which were in the form of thick bands, and were densely packed and well organized, with strong birefringence (Figure 1B). In comparison, in discohesive tumors, front collagen fibers were thin, loosely packed, and disorganized, with weak birefringence, probably due to type III collagen fibers (Figures 2B, 3, and 4). This is consistent with Junqueira et al. and Montes et al.'s concept that the thick fibers were type I collagen fibers and exhibited an intense birefringence of red, orange, and yellow by polarizing microscope, and a weak birefringence of green when fibers were thin fibrillar, thus consisting of type III collagen.[16,17] This is further supported by Stenback et al., who found that the presence of a delicate meshwork (reticular) of type III collagen at the invading front of tumor islands increases gradations of skin cancer.[18] In a study on stromal reaction during tumor progression in oral mucosa, Yokoyama revealed that type I collagen stained red by PSR decreased with advanced dysplastic grading.[19]

Thus, the color changes observed in the present study clearly indicate some alteration in the stromal tissue around the tumor island

of the advancing front, which could be due to carcinogenic agents involved in tumorigenesis. The above results are further supported by Brekken et al., who stated that the tumor progression is influenced by ECM.[20] This finding is further supported by a study on the mechanics of capsule formation, which revealed that a more robust ECM and capsule results in slower tumor growth.[21]

Further, nuclear resonance studies on the physical aggregation of collagen fibers by Sharf et al. also revealed a color profile of orange to red while corresponded to the well packed fiber and green to greenish yellow to poorly packed fibers.[22] This collagen could have originated from the tumor cell, thus benefiting the tumor by reducing access to host lymphocytes.

Alternatively, the collagen could have stromal cell origin, thus benefiting the host by walling off the invading tumor. The results in the present study also revealed that, in nine cases (53%), tumor size <4 cm showed densely-packed collagen fiber, and in 10 cases (83%), tumor size >4 cm showed loosely-packed collagen fibers around the tumor island of the advancing front. Therefore, tumors with excessive collagen in the stroma could respond in this manner, as seen in a study on breast cancer, where an increase in the collagen content of ECM was found to increase mechanical stiffness and transport resistance of tumors.[23] A study on myocardial infarction concluded that collagen degradation and loss after myocardial infarction is associated with infarct expansion, followed by functional decline.[24]

Previous literature has confirmed that invasive front grading, in contrast to Broder and Bryne's grading, is of high prognostic value, because of the following reasons: (a) tumors often are more poorly differentiated in invasive parts compared to superficial parts;[25] (b) blood group H antigen is often lost in invasive tumor margins of OSCC, and this loss is associated with poor prognosis;[26] (c) the increased expression of proliferation-associated structures in the invading tumor front;[27] (d) melanoma cells from deep tumor parts have a higher DNA content than more superficial cells;[28] (e) the increased expression of Ki-67, L-myc, c-myc, N-myc, and c-erbB-2 oncoproteins in most invasive lung cancers;[29,30] and (f) the increased labeling of bromodeoxyuridine at the site of invasion.[31]

All of these studies show that the key to better understanding the invasive and metastatic behavior of cancer cells could reside within the invasive margins of different tumors. Therefore, in the present study, collagen fiber nature was analyzed with modified invasive front grading.

As proposed by Bryne et al.,[32] the pattern of invasion is divided into four grades: (a) grade I: pushing, well-delineated infiltrating borders; (b) grade II: infiltrating, solid cords, bands, and/or strands; (c) grade III: small groups or cords of infiltrating cells (n>15); and (d) grade IV: marked and widespread cellular dissociation in small groups and/or in single cells (n<15). The cases were divided into two groups: the majority (n=19, 63%) of tumors showed a "discohesive tumor front" represented by grades II, III, and IV (66%), followed by a "cohesive tumor front" in 11 tumors (37%), which represented grade I.

With respect to the relationship between the collagenous component in the stroma and the invading tumor front, observable changes with different advancing tumor fronts (i.e. cohesive and discohesive) could be seen in the present study.

The present study also revealed that the majority of cases, which were graded as TNM stages I, and II (n=9, 56%), with the absence of lymph node metastasis (n=10, 53%), showed densely-packed collagen fibers, whereas in stages III and IV tumors (n=11, 85%) with evident metastasis (n=8, 89%), collagen fibers were loosely packed.In their study on stromal differences in salivary gland tumors, Alon et al. found that 50% of collagen fibers in polymorphous low-grade adenocarcinomas and adenoid cystic carcinomas were greenish-yellow, whereas in pleomorphic adenomas, only 13% were greenish-yellow.[33] Similarly, in a study on skin and lower lip squamous cell carcinoma, it was revealed that high-grade tumor cell dissociation, represented by a spray-like pattern of invasion, was significantly associated with a high frequency of metastatic disease, as well as recurrent disease.[34,35] It was also reported that a non-cohesive (spray-like) pattern of invasion was significantly associated with lymphovascular space involvement and large tumor size.

Contrary to our results, collagen fiber nature was not significant with Broder and Bryne's grading system.As discussed in Kalele and Venigella's and studies, collagen fibers in well-differentiated carcinoma revealed polarizing reddish-orange colors around the tumor island, which gradually changed into yellow-orange in moderately-differentiated carcinoma, and greenish-yellow with weak birefringence in poorly-differentiated carcinoma. However, this was associated with invasive front grading, according to by Bryne et al.,[36] Previous literature has also confirmed that invasive front grading in contrast to the conventional Broder and Bryne's grading is of high prognostic value. Irrespective of different scoring systems, the common findings of all the studies was that a discohesive tumor front was associated with metastasis, large tumor size, advanced tumor stage (TNM stages III and IV), increased recurrence, and decreased survival.

In the present study, an observable change in collagen with different patterns of invasion was revealed with the pattern of invasion. Adjacent to the cohesive tumor front represented by pushing borders of invasion is positive correlated with thick bands of collagen which were well organized, and resist the tumor against invasion and metastasis, preventing it to increase in size and thus associated with initial stage of tumor (I & II).In the discohesive tumor front, the fibers are thin and disorganized, which could increase movement of tumor cells toward invasion and metastasis.

Although PSR stains very thin collagen fibers in comparison to other collagen stains, factors, such as pH, the concentration of staining, and the duration of staining, will lead to variations in results.

Stain samples can deteriorate when kept for more than 4 years.The solution loses its specificity, and besides staining collagen, it also stains muscle and epithelia.[12]

It is not advisable to use the staining technique on tissue preserved in formalin for a number of days. Thus, researchers must aim at ultrastructural features of connective tissue in different stages of OSCC in future.

4.1 | Conclusion

Based on the results of the present study, it can be concluded that PSR staining with the use of a polarizing microscope is the most

suitable staining to visualize collagen fibers. Staining application is a relatively simple tool to study the changes in the ECM, in particular the structural integrity of collagen fibers at the different invasive front of OSCC.

Determination of collagen fiber nature in different patterns of invasion of oral squamous cells can help target the stroma for various treatment strategies. Further research with larger sample sizes is warranted. Immunohistochemistry and collagen gene identification, second harmonic generation microscopy, and confocal laser microscopy are advanced techniques that could be used specifically for collagen detection.

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